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Genomic Analysis of 727 Patients with Ehlers-Danlos Syndrome I: Clinical Perspective Relates 23 Genes to a Maternally Influenced Arthritis-Adrenaline Disorder

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Abstract

A novel medical approach for qualifying DNA variants found by whole exome sequencing (WES) facilitates discovery of new gene-disease relationships and emphasizes that DNA change must be correlated with clinical findings before having utility for diagnosis. Delineation of an arthritis-adrenaline disorder (AAD) process qualified variants in 23 genes as diagnostically useful in 727 patients having WES among 1656 with Ehlers-Danlos syndrome (EDS); these results distinguished them from 102 patients who had qualified gene variants among 728 with developmental disability. Excess maternal transmission of AAD by pedigree analysis plus 167 maternally versus 111 paternally transmitted DNA variants and 75 patients with only mitochondrial DNA variants suggest maternal influence on inheritance of AAD and its subsumed EDS types. Genes grouped by impact on different connective tissue elements showed variation in similar numbers of patients with hypermobile or classical EDS, benign joint hypermobility, or predominant dysautonomia: *COL7A*1, *FLG* acting on skin in 21 patients; *SCN*9*A*/10*A*/11*A*, *POLG* on nerve in 24; *COL*6*A*1/*A*2/*A*3, *COL*12 on muscle in 19; *COL*5*A*1/*A*2, *FBN*1, *TGFB*2/3, *TGFBR*1/2 on tissue matrix in 51; *COL*3*A*1, *VWF* on vessel in 18; *COL*1*A*1/*A*2, *COL*11*A*1/*A*2 acting on bone in 15 patients. Each gene group acts through a postulated articulo-autonomic dysplasia cycle to produce reciprocal tissue laxity and dysautonomia findings that transcend EDS types. This same tissue laxity-dysautonomia cycle acts to produce secondary complications in disorders ranging from distinctive connective tissue dysplasias to developmental disorders with hypotonia and acquired conditions with autonomic imbalance. Several altered genes were previously associated with neuromuscular disorders, foreshadowing a large myopathic EDS category that

will incorporate many patients with hypermobility. The importance of muscle for joint constraint supports present exercise and future mesenchymal stem cell therapies, whether AAD is genetic or epigenetic from trauma, surgery, inflammation, or aging.

Keywords

Ehlers-Danlos Syndrome, Connective Tissue Dysplasia, Arthritis-Adrenaline Disorder, Articulo-Autonomic Dysplasia, Whole Exome Sequencing, Collagen Genes

1. Introduction

"What signifies knowing the Names, if you know not the Nature of Things?" [1]

Few preventive health care opportunities are more powerful or less appreciated than those that attend recognition of tissue flexibility [2]. The approach from general to particular, so essential for Linnaean taxonomy and medical nosology, would easily apply to hypermobility had not extreme phenotypes like Ehlers-Danlos syndrome (EDS) become iconic for a trait that affects 10% of males and 20% of females [2] [3] [4] [5]. Early and current focus on unusual findings like circus-worthy skin elasticity [6] [7], piezogenic papules [8], or aneurysms [9] has promoted a view of rare and discrete types [2] of EDS that underestimates its considerable prevalence and spectrum quality within the larger category of connective tissue dysplasia (CTD) [10]. Even worse for many with CTD is neglect of that hypermobility cohort in crime, autonomic imbalance [11] [12] that leads to treatable complications like irritable bowel syndrome [13], postural orthostatic tachycardia [14], and mast-cell activation disorder [12] [15] [16].

A previous report [17] suggested articulo-autonomic dysplasia as a disease process and arthritis-adrenaline [18] disorder (both AAD) as a preliminary diagnosis for patients presenting with tissue laxity, hypermobility, and autonomic balance characteristic of EDS, explicitly recognizing the adrenergic compensation that counteracts vessel distensibility/lower body blood pooling to restore cerebral circulation. AAD becomes the genus that can initially subsume common EDS types and synecdochic diagnoses like fibromyalgia [19], anxiety disorder [20], or chronic fatigue syndrome [21] when they are applied to these patients. AAD is less inclusive of diseases like vascular EDS (M130050) [22] or Marfan syndrome (M154700), usually differentiated by focal findings like aneurysms and bowel ruptures or lens dislocation and aortic dilatation but can be anticipated as a secondary complication when these diagnoses are considered. It can also occur in genetic disorders ranging from Down syndrome to skeletal dysplasia, and of multifactorial neurologic and inflammatory conditions that

precede or accompany the inevitable decline of aging [23] (see Discussion).

Perfectly positioned to liberate EDS from specious rarity and misdiagnosis are the advances in NextGen or massive parallel sequencing that allow screening of all human genes rather than a select few. Genomic screening for changes in DNA dosage (microarray analysis) [24] or in DNA [24] [25] [26]/RNA [27] sequence is transforming a monogenic view of genetic disease into one of polygenic networks and processes. Freed from the focus of targeted analysis, NextGen sequencing focused on the translatable genome (whole exome sequencing or WES) [25] [26] is proposing candidate genes in neurologic [24] [28] and connective tissue dysplasia (CTD) [29] that deserve electoral scrutiny by informed practitioners, yet leaders of mainstream medicine proclaim that the wish for DNA-guided precision medicine has not been fulfilled [30].

While the latter bundling of DNA variant association [31] and pathogenic coding change [25] [26] is short-sighted, DNA rejection because laboratories short-circuit physician translation of results into management is reasonable. This article outlines a novel clinical approach to DNA variant qualification that relates new genes to AAD and EDS but is applicable to any complex disease process.

2. Methods

Reported here are patients referred for evaluation of EDS from January 2011 to June 2018 when ordering whole exome sequencing [25] [26] became practical via preliminary ascertainment of insurance coverage by the GeneDx© Company. Systematic evaluations [17] using forms based on common findings in 946 patients were used for 710 referred after September 2016. Different evaluations of 728 patients with developmental disability and/or autism were performed over the 2011-2016 time period, employing separate microarray and WES analyses by GeneDx before their combined technology [32]. Provisional clinical diagnoses following criteria for hypermobile hEDS (more dramatic hypermobility leading to subluxations and joint injuries along with elastic, velvety skin) [4], classical cEDS (milder joint issues, elastic and fragile skin with typical scarring) [8], benign joint hypermobility (hypermobility with minimal skeletal complications) [4], or dysautonomia (autonomic imbalance out of proportion to skeletal issues) [11] [12]. A minority of this informed and/or referred population were told that they did not meet EDS criteria and 12 patients who had obvious diagnoses like Marfan syndrome are not included in this study. No typical cases of vascular EDS (M130050) [9] were recognized.

GeneDx uses standard methods for whole exome sequencing [25] [26] with recent additional detection of deletions or duplications involving three or more coding exons [32]. Their reports first qualify sequence variants as "variants in disease genes associated with reported phenotypes" or those "possibly associated" according to consensus gene-disease relevance guidelines [33], also reporting "secondary findings" [34] for those patients giving consent. They then

use consensus qualification guidelines [35] to report variants as pathogenic, likely pathogenic, or variants of uncertain significance (VUS)—variants deemed benign or likely benign, mostly single nucleotide polymorphisms associated with ancestry or susceptibility [31], are not reported by GeneDx. Over time a novel approach to DNA variant significance interpretation was developed that relied heavily on clinical experience with EDS (see Results).

Patients and/or families were given forms to consent for medical genetic evaluation/treatment and anonymous sharing of DNA results from whole exome sequencing (WES) during patient intake, counseled using EDS as the recognized diagnostic term while explaining the concept of AAD, offered discussion of ambiguous, incomplete, and incidental/secondary WES findings [34], consented to send their insurance information to GeneDx for estimates of out-of-pocket costs, and provided hand-outs containing management information at the end of the 60 to 80-minute outpatient visit. Interaction with GeneDx mediated through their senior genetic counselors included obtaining and negotiating out-of-pocket cost estimates for testing using the ICD10 code Q79.6 for EDS, other codes for those with developmental delay and/or autism.

Cost estimates for standard patient-parent trio WES plus mitochondrial DNA testing (parents for reference only) varied with deductibles but partial data on two-thirds of the 727 EDS patients having WES testing indicates that around 45% requesting information received estimates of $0 out-of-pocket, 6% of $10 to $500, 5% of $550 to $1000, 14% of $1050 to $3000 ($2500 is the current self-pay cost at GeneDx), 30% of more than $3000, many of the latter negotiated downward by review of family/income circumstances. A very small number of insurance requests for records indicated that they were billed around $20,000 for WES, but no information on actual payments by patients or insurance reimbursements to GeneDx is available. For those electing to proceed, the GeneDx counselor completed requisitions that contained a second consent for de-identified data-sharing as well as an option to be informed of secondary findings and sent them to patients with kits for blood or cheek swab sampling of patient/parent trios. Results were conveyed by fax and/or internet portal after an average 5.1 months (2011-2014) to 3.1 months (2015 on).

GeneDx reports with interpretative physician letters were mailed to families and included options for follow-up discussion and testing of relatives, the latter again coordinated by the GeneDx counselor. Historical, physical, and molecular findings were abbreviated and entered into a password-protected MS Excel© database after IRB approval; tallies of findings and statistical analyses were performed using standard Excel formulae or online calculation [36].

3. Results

Inheritance. Figure 1 shows the relatives of 1219 females and 226 males evaluated for EDS (87% of 1656 patients) who provided sufficient family history information, female patients centering the pedigree diagram as probands (arrows)

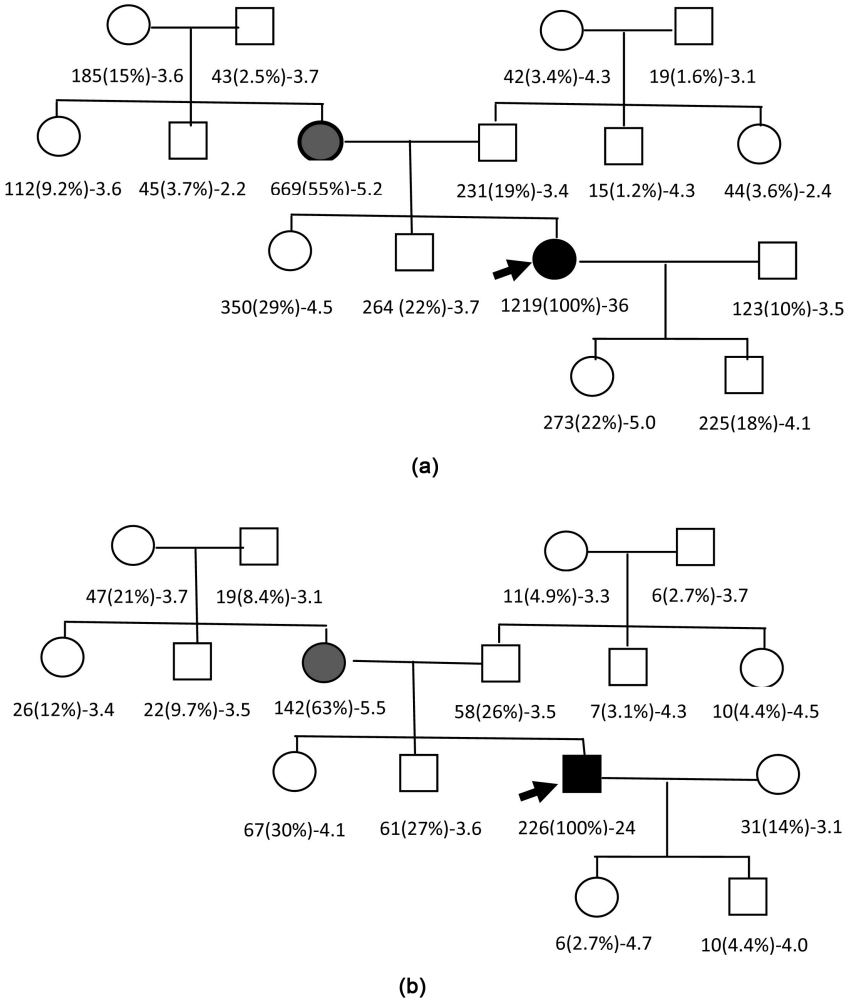

Figure 1. Affected relatives of AAD females (a) and males (b). Patients are shown as probands (arrows), relatives as standard pedigree symbols, their numbers, (percentages), and average number of findings (after the dash) shown beneath the symbols. All female versus male relative differences for female probands but only maternal grandparent and parent differences for male probands were significant by chi square analysis, 1 degree of freedom, $p < 0.05$.

in **Figure 1(a)**, males as probands in **Figure 1(b)**. Numbers of probands and their relatives with two or more AAD findings [17] are shown beneath their pedigree symbols, exemplified by the 1219 female probands in **Figure 1(a)** who had 669 mothers (55% of their mothers). Average numbers of AAD findings in probands or relatives, much higher for the former who presented for evaluation, are listed after the dash (e. g., 36 for female probands and 5.2 for their mothers, **Figure 1(a)**). These 1219 female probands had fewer symptomatic fathers, 231 of 19% of their fathers, their fewer average findings (3.4) reflecting lesser AAD severity in most males [17]. The 226 male probands also had a higher percentage of their mothers (63%) than fathers (26%) with more than 2 AAD findings (**Figure 1(b)**).

Higher numbers of female relatives with AAD is a general trend, shown not only by the 185 maternal grandmothers versus 43 grandfathers, the 42 paternal

grandmothers versus 19 grandfathers, the 112 maternal aunts versus 45 uncles, the 44 paternal aunts versus 15 uncles, the 350 sisters versus 264 brothers of female probands in **Figure 1(a)** but by the respective 47 versus 19 and non-significant 11 versus 6, 26 versus 22, 10 versus 7, 67 versus 61 for male probands in **Figure 1(b)**. Supporting a role for autosomal dominant inheritance in many cases of AAD as accepted for subsumed EDS types were 279 families overall (120 having standard evaluation) in whom members of 3 generations had 2 or more typical findings (data not shown). The 10 of 20 example patients in **Table 1** who inherited a DNA variant from their parent (mat for mother, pat for father) also support frequent dominant inheritance of AAD and EDS.

The excess of affected female relatives not only reflects greater female expression of AAD but also shows preferential female transmission since female AAD probands have significantly more symptomatic daughters (273) than sons (225—**Figure 1(a)**) while male AAD patients (6 and 10, respectively, **Figure 1(b)**) do not. The similar proportions of daughters and sons regardless of proband sex, though daughters may be over-represented because of greater expression, argues for maternal rather than X-linked inheritance, especially because more sons (10) than daughters (6) were born to male probands (**Figure 1(b)**) though the difference is not significant. Also unexpected were the 10% of female proband and 14% of male proband spouses with 2 or more findings, averaging a respective 3.5 versus 3.1 findings per spouse. This along with the significant numbers of AAD and EDS patients seen in a 7-year period supports a greater prevalence of EDS than is commonly acknowledged and promotes expectation of polygenic inheritance.

Interpretation of DNA diagnostic utility. The novel approach in **Figure 2** restores medical guidance to DNA variant interpretation by emphasizing clinical knowledge of disease process and symptom pattern, here focused on the AAD pattern as defined previously [17]. Current guidelines look at patient symptoms to decide if a gene variant: 1) travels more with the disease in question [33] than ostensible health [37], and 2) changes encoded RNA/protein structure sufficiently to warrant qualification as pathogenic [35] and "diagnostic" [38]. **Figure 2** modifies this approach by determining: 1) structural disruption (column D) [39] of the DNA-directed protein change in light of that protein's role in disease, here recognizing the key roles of glycine and proline in the collagen triple helix [40]; 2) relevance of a gene and/or gene family to general symptoms of a disease *process* (columns G, H) rather than to those of a *specific disease*, here looking at AAD rather than a particular type of EDS; 3) qualifying DNA variants by diagnostic *utility* for the disease process (column V*DU), recognizing that molecular change, like any laboratory test, must be correlated with family context (column I) and specific disease symptoms (column Fd) before it can suggest a clinical diagnosis (columns IFClin in **Figure 2**). The approach in **Figure 2** honors the long-established role of physicians in translating laboratory results into patient diagnosis and management.

Table 1. Example patients with gene variants of definite relevance to AAD.

Pt	Sex	Age (y)	Hx-PE[a]	JtSnFlex[b]	DysA[c]	PreDx[d]	DNA variant,[e] source,[f] GeneDx[g] and author[h] qualifiers, prior occurrence[i]	ClinDx,[j] tissue impact/systems involved,[k] prior disease association[l]
1	f	13.7	48-20[a]	9-4-7[b]	14[c]	c[d]	*COL*1A1 p.Pro982Thr c.2944C>A,[e] mat[f] VUS[g] VSDU-3+[h] Vi5 (LkPath-1)G1H1[h] rs141117382-2pt[i]	AAD-cEDS,[j] -**Oss**-Er-CVS-Nm[k] *COL1A1* M120050 a/w OI types 1-IV M166200+[l] EDS cardiovascular M225320+;[l] utility increased by the sodium channel M601827 gene variant a/w atrial fibrillation-14 M615378; the potassium channel, voltage-gated type II, subfamily H, member 2 M152427 gene variant a/w long QT syndrome-2 M613688 suggests dual diagnoses (+ arrhythmia).
							KCNH2 p.Arg1005Gln c.3014 G>A pat VUS VCDUO Vi4 (VUS-0)G1H1 rs199473019-1pt	
							SCN2B p.Arg28Gln c.83G>A patSx VUS VSDUS Vi5 (LkPath-1)G1H1 rs72544145-1pt-Path-atrial fibrillation	
2	m	14.4	23-16	6-1-9	6	h	*COL1A2* p.Arg432Gln c.1295G>A unknown VUS VSDU-3+ Vi5 (LkPath-1)G1H1 rs139446305-3pt-1LkPath-EDS	AAD-hEDS-**Oss**-Er-CVS *COL1A2* M120160 a/w OI types II-IV M166210+, arthrochalasia EDS M617821+; utility increased by the collagen type XV M120325 gene variant, no disease correlation yet but likely with AAD as a collagen gene.
							COL15A1 IVS1 6T>G c.12 6T>G matSx VUS VSDUS Vi6 (LkPath-1) G1H1 new	
3	f	19.9	44-15	10 - 4-9	13	h	*COL11A1* p.Leu654Pro c.1961T>C patSx broSx VUS VADU-4+ Vi6 (LkPath-1)G1H2 rs1131691449-1pt	AAD-mEDS-**Oss**-Ey-Nm *COL11A1* M120280 a/w Marshall M154280 and Stickler M604841 syndromes; utility increased by the myosin heavy chain 2 gene M160740 variant a/w myopathy and ophthalmoplegia M605637
							MYH2 p.Val102Met c.304G>A unknown broSx VUS VCDUS Vi5 (LkPath-1)G0H1 rs1131691454-1pt	
4	f	38.4	36-22	6-4-8	14	h	*COL11A2* p.Arg1020Ter c.3058C>T trans patSx LkPath VADU-4+Vi6 (LkPath-1) G1H2 rs911722283-1pt-1LkPath-?disease	AAD-hEDS-**Oss**-Ey *COL11A2* M120280 skeletal dysplasia M614524+ and hearing loss M601868+; utility increased by additional *COL11A2* gene variant
							COL11A2 p.Arg1551Gln c.4652 G>A trans matSx VUS VADUS Vi6 (LkPath-1)G1H2	
5	f	36.8	43-19	8-3-11	14	h	*VWF* p.Arg854Gln c.2561G>A unknown LkPath VADU-4+Vi6 (LkPath-1)G2H1rs41276738-10pt-9Path-von Willebrand disease	AAD-hEDS-**Vss**-Heme *VWF* M613160 von Willebrand factor a/w von Willebrand diseases (vWD) M193400+[l]
6	f	34.7	40-24	8-1-10	16	h	*COL3A1* p.His34Arg c.101A>G unknown VUS VCDU-2+Vi2 (VUS-0)G1H1 rs752110396-2pt	AAD-hEDS-**Vss**-CVS *COL3A1* M120180 a/w vascular EDS M130050 and polymicrogyria M618343
7	f	0.2	7-7	0-1-6	3	n	*COL5A1* p.Asp1761Gln c.5281G>A unknown Path VCDU-2+Vi5 (LkPath-1)G1H0 new	AAD-hEDS-**Mtx-**Epi
8	f	19.1	43-24	7-4-8	15	h	*COL5A2* p.Gly126Ser c.376 G>A unknown VUS VCDU-2+Vi3 (VUS-0)G1H1 rs779153546-2pt-1LkPath	AAD-cEDS-**Mtx-**Epi *COL5A2* M120190 a/w classical EDS-2 (M130010)[l]

Continued

9	f	10.2	28-13	6-1-8	6	h	*FBN*1 p.Leu925Val c.2773C>G patSx VUS VSDU-3+Vi2 (VUS-0)G1H2 rs149681175-1pt *HFE* p.Cys282Tyr c.845G>A homozygous mat/pat Path VADUO Vi7 (LkPath-1)G2H1 rs1800562->10pt-Path-hemochromatosis *MYBPC*3 p.Val757Met c.2269G>A matSx LkPath VADUOVi7 (LkPath-1)G1H2 rs369790992-5pt	AAD-hEDS-**Mtx**-Ey-CVS-Dig *FBN*1 M134797 a/w Marfan 154,700+, skeletal dysplasia M102370+; the *HFE* (M613609) gene variant a/w hemochromatosis-1 M35200 and susceptibility for porphyria cutanea tarda M176100 suggests dual diagnoses (+ hemochromatosis carrier)
10	f	60.3	36-12	8-4-5	11	h	*TGFB*2 p.Ile239Phe c.715A>T unknown VUS VSDU-3+Vi5 (LkPath-1)G1H1 rs1131691445-1pt-Path-LDS4	AAD-hEDS-**Mtx**-CVS-Pul *TGFB*2 M190220 a/w Loeys-Dietz syndrome-4 (LDS4) M614816
11	f	38.8	42-16	8-3-10	14	h	*TGFB*3 IVS4 (intron4)-1G>C c.755-1G>C unknown VUS VADU-4+Vi8 (Path-2)G1H1 new	AAD-hEDS-**Mtx**-CVS-Sk *TGFB*3 M190230 a/w LDS5 M615582 and arrhythmogenic RV dysplasia M107970
12	f	41.5	30-16	2-3-4	14	c	*TGFBR*1 p.Tyr291Cys c.872A>G matSx VUS VADU-4+Vi6 (LkPath-1)G1H3 new *FLG* p.Arg501Ter c.1501C>Tunknown Path VADUS Vi6 (Path-2)G2H1 1.6% rs61816761-5pt-4Path-ichthyosis *FLG* p.Ser3247Ter c.9740C>A matSx Path VADUS Vi6 (LkPath-1)G1H2 rs150597413-1pt-1Path	AAD-cEDS-**Mtx**-CVS-Epi-Sk *TGFBR*1 M190181 a/w LDS-1 M609192; utility increased by the profilaggrin M135940 gene variants a/w ichthyosis vulgaris M146700 and atopic dermatitis-2 M605803
13	f	18.1	41-18	8-3-9	13	h	*TGFBR*2 p.Glu151Val c.452A>T matSx VUS VADU-4+Vi6 (LkPath-1)G1H2 new	AAD-hEDS-**Mtx**-CVS-Di-Sk *TGFBR*2 M190182 a/w LDS2 and colorectal cancer 614331
14	f	36.6	47-9	7-2-4	17	c	*SCN9A* p.Glu1129Asp c.3387A>T unknown VUS VSDU-3+Vi5 (LkPath-1)G1H1 new	AAD-mEDS-**Nrv**
15	f	35.8	30-12	3-1-6	13	c	*SCN*10*A* p.Val1617Phe c.4849G>T matSx VUS VADU-4+Vi6 (LkPath-1)G1H2 rs375940680 - 4pt	AAD-mEDS-**Nrv** *SCN*10*A* sodium channel type X α-subunit M604437 a/w familial pain syndrome-2 M615551[1]
16	f	45.9	30-13	7-2-7	12	c	*SCN*11*A* p.Gln367Arg c.1100A>G unknown VUS VSDU-3+ Vi4 (VUS-1)G1H1 new	AAD-mEDS-**Nrv** *SCN*11*A* sodium channel type XI, α-subunit M604385 a/w HSAN VII M615548+
17	f	36.8	39-16	8-2-9	15	h	*POLG* p.His277Leu c.830A>T unknown Path VADU-4+ Vi6 (LkPath-1)G2H1 rs138929605-6pt-1LkPath	AAD-mEDS-**Nrv**-Nm *POLG* M174763 gene variant a/w mitochondrial depletion/MNGIE-4B M613662+
18	m	16.9	36-12	7-0-7	12	h	*COL6A*1 p.Thr214Met c.641C>T patSx VUS VADU-4+ Vi6 (LkPath-1)G1H2 NM_001848.2-1pt	AAD-mEDS-**Nrv** *COL6A*1 M120220 a/w Bethlem myopathy-1 M158810, AR Ulrich dystrophy-1 M254090
19	f	31.4	19-16	3-0-6	7	d	*COL6A*2 p.Arg317His c.950G>A patSx VUS VADU-4+ Vi5 (LkPath-1)G1H2 rs373782637-1pt	AAD-mEDS-**Nrv**-Nm *COL6A*2 M120240 a/w Bethlem myopathy-1 M158810, AR Ulrich dystrophy-1 M254090

Continued

20	f	56.5	42-17	8-2-10	15	h	*COL6A3* p.Ala876Val c.2627C>T unknown VUS VADU-4+ Vi8 (Path-2)G1H1 NM_004369.3-1pt	AAD-mEDS-**Nrv**-Nm *COL6A3* M120250 a/w Bethlem myopathy-1 M158810, AR Ulrich dystrophy-1 M254090
21	f	17.1	37-17	7-1-8	12	h	*COL12A1* p.Thr826Met c.2477 C>T patSx VUS VADU-4+ Vi5 (LkPath-1)G1H2 new	AAD-mEDS-**Nrv**-Nm *COL12A1* M130230 a/w Bethlem myopathy-2 M646471;
22	f	42.0	41-22	8-2-9	14	d	*COL7A1* p.Gly1568Ser c.4732G>A matSx Path VADU-4+ Vi6 (LkPath-1)G1H2 new	AAD-hEDS-**Cut** *COL7A1* M120120 a/w epidermolysis bullosa M132000+;
23	f	17.3	28-22	3-2-9	11	h	*FLG* p.Arg501X c.1501C>T pat Path VADU Vi8 (Path-2)G2H0 1.6% rs61816761-pt5-Path4-ichthyosis	AAD-hEDS-**Cut** *FLG* filaggrin M135940 a/w ichthyosis M146700 and atopic dermatitis-2 M605803

[a]Total numbers of historical (Hx)-physical (PE) findings by standard evaluation reported previously [17]; [b]average numbers of findings in the joint-skeletal (JtSkt out of 15 total)-skin by history (Sn out of 5)-flexibility (Flex out of 12 including Beighton score with 9 plus 3 other maneuvers) categories;; [c]average number of findings in the dysautonomia (DysA) category out of 20; [d]preliminary diagnosis (PreDx) before DNA testing (h, hypermobile or c, classical EDS, b, benign joint hypermobility, d, dysautonomia, n, not EDS); [e]variant as defined by protein (p.) change--fs, frame shift, X, terminating mutation, amino acid codes in Grantham reference [39]; [f]source—inheritance unknown or from mother/father/brother (mat/pat/bro) with AAD (arthritis-adrenaline disorder), symptoms (Sx) if 2 or more AAD findings are present [17]; [g]GeneDx© qualification reported as VUS, variant of unknown significance, LkPath, likely Pathogenic, Path, Pathogenic; [h]author qualification from **Figure 2** showing diagnostic utility for primary variant (the variant deemed most relevant to AAD) as V*DU with V, variant, * for U uncertain-1+, C conditional-2+, S strong-3+, A, actionable-4+ diagnostic utility (DU), qualifying additional variants as having synergistic (V*DUS) or divergent/other (V*DUO) action, followed by variant impact (Vi) (0 - 1, 2 - 4, 5 - 7, 8 - 10 qualified as Lkbenign, VUS, LkPath, Path, contributing 0, 0, 1, 2+ respectively), gene-relevance (G) contributing 0 - 2+, history correlation (H) contributing 0 - 2+ to the preceding 0 - 4+ diagnostic utility qualification; [i]prior occurrence as new (variant not seen before), rs or NM numbers with number of patients (pts) having variant plus qualification as VUS, etc. [37], number with % indicating variant prevalence over 1%--no listing indicates that the variant was observed before with a prevalence below 1 in 1000 [37]; [j]clinical diagnosis (ClinDx) after DNA testing and author interpretation; [k]connective tissue element most impacted (**Cut**, cutaneous, **Mtx**, joint-tissue matrix; **Mus**, muscle, **Nrv**, nerve, **Oss**, cartilage-bone; **Vss**, vessel) followed by systems especially at risk (CVS, cardiovascular, Epi, epidermal, Er, hearing, Ey, eye, Di, digestive, Heme, hematologic, Im, immunologic, Pul, pulmonary, Nm, neuromuscular, Sk, skeletal); [l]prior disease associations with M numbers from www.omim.org referencing genes and diseases, + indicating several associated diseases; AR, autosomal recessive; a/w, associated with; CTD, connective tissue dysplasia; LDS, Loeys-Dietz syndrome; MNGIE, mitochondrial neurogastrointestinal encephalopathy; OI, osteogenesis imperfecta.

Table 1 provides examples of this approach, beginning with patient #1 having a DNA variant that changes proline (Pro) to threonine (Thr) at amino acid position 982 of the collagen type I alpha-1 (COL1A1, M12005) protein (variant symbolized as p.Pro928Thr with corresponding DNA change as c.2944C>A = Cytosine to Adenine at nucleotide #2944). This amino acid substitution has a low Grantham score (38 compared to the 215 maximum) [39] with indecisive evolutionary (column E, **Figure 2**) or functional (column Fa) changes and was reported as a variant of uncertain significance by Gene Dx (**Table 1**, upper row). Clinical knowledge that proline accounts for 1/3 of the amino acids in fibrillar collagens and the importance of vitamin C-promoted proline hydroxylation for wound healing [40] assigns a variant impact score (Vi in **Figure 2**) of 5 and likely pathogenic qualification [35] in **Table 1**.

More important for clinicians are the next steps in qualifying DNA variants because they can accept judgments of variant impact from whole exome sequencing (WES) laboratory reports much as they would designation of outlying values from a comprehensive metabolic panel. Relevance of the DNA variant to disease follows biological and nosologic principles by looking first at genus (disease process/category like arthritis-adrenaline disorder) rather than species

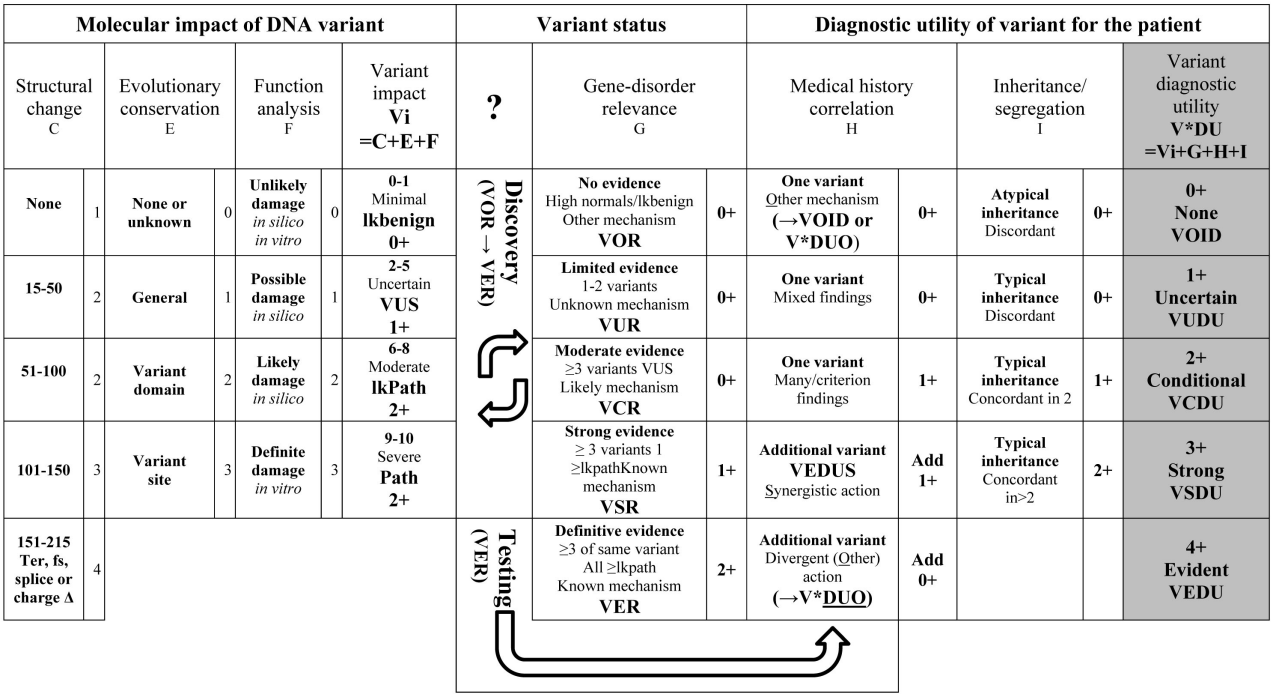

Figure 2. Scoring of DNA sequence variants for diagnostic utility. Degree of protein structural Changeis rated 0 for synonymous mutations or according to Grantham scores (15 - 215)[32] for missense variants, also assigning 4 points for terminating (Ter) and frameshift (fs) variants leading to nonsense-mediated mRNA decay, variants that interfere with RNA splicing (splice), or differences (Δ) in aminoacid charge not accounted by Grantham (for genes encoding proteins like collagen III where missense mutations may disrupt function more than nonsense ones, 2+ is assigned for the latter—RNA changes can be assigned similar points according to regional structural importance, e. g., loop regions in transfer RNA); Vi, variant impact scores are obtained by adding C, E, and Fpoints, translating to consensus qualifications[28] (lkbenign, likely benign, lkpath, likely pathogenic, path, pathogenic, VUS, variant of uncertain significance); ? indicates branch point for scoring of variants with established relevance (VER) to the disease in question (use of DNA sequencing for testing) or scoring of new variants (use of DNA sequencing for discovery) as (V*R) for relevance to disease where * is O for none, U for uncertain, C for conditional, S for strong, E for established; variants of high frequency in individuals judged unaffected by experienced clinical evaluation (normals), [34]scored as benign for most occurrences, or having a different disease mechanism are qualified as VOR; to qualify for many/criterion findings (1+) under the medical history correlation (H) at least 30 historical and 10 physical findings of AAD are required; additional variants[33] with Synergistic action (V*DUS), divergent action (V*DUO, DUO connoting dual diagnoses for higher utility scores, VOID, Variant for Other than Indicated Diagnosis for lower ones) may add pluses to the final diagnostic utility (V*DU) qualification for the particular patient, where * is the same as for V*R above except that E is for evident since utility for one person does not establish utility for all.

(component conditions like classical or hypermobile EDS), recognizing that *COL*1*A*1 gene variants like that in patient #1 have been seen in EDS as well as osteogenesis imperfecta (M166200, etc.) [41]. Just as bacteria associated with different pathology (e.g., impetigo versus furuncles) can be relevant to a disease process like septic shock, so can genes long associated with articular laxity (e.g., collagen type V *COL*5*A*1, M120215 in patient #7, **Table 1**) versus those with autonomic imbalance (e.g., DNA polymerase gamma *POLG*, M174763 in patient #17) become relevant to EDS when the subsuming AAD process is appreciated.

Qualification of relevance follows usual guidelines [33] where 2 - 3 variants in a particular gene garner possible (uncertain-VUR, conditional-VCR, **Figure 2**) and 3 or more with pathogenic disruption likely relevance (strong-VSR or estab-

lished-VER for identical variants): Once qualified, gene relevance and variant impact scores are added to produce 0 - 4+ diagnostic utility scores V*DU, * conveying the same modifiers as above except that A for actionable is substituted for E since scoring in one patient does not *establish* diagnostic utility in all.

The von Willebrand (*VWF*) gene variant in patient #5 of **Table 1** was rated as likely pathogenic (Vi = 6, adding 1+ to the utility score) and relevant to AAD rather than von Willebrand disease as reported by GeneDx because 8 other AAD patients with *VWF* gene variants have been observed (**Table 2** and **Table 3**). The 10 identical p.Arg854Gln qualifications as pathogenic (patient #5 plus 9 in the ClinVar database, **Table 1**) [35] added 2+ for established relevance (column G, **Figure 2**) while the 43 of 80 historical and 19 of 40 physical findings typical of AAD (left columns, **Table 1**) added 1+ (column H, **Figure 2**), totaling a 4+ or VADU qualification of diagnostic utility. High numbers of specific clinical findings (left columns, **Table 1**)--joint-skeletal (8 of 15), skin (3 of 5), and hypermobility (Beighton plus 3 others—11 of 12) plus dysautonomia (14 of 20) supported translation of the high diagnostic utility into a clinical diagnosis of hypermobile EDS (right column, **Table 1**). This implies usual management for AAD and EDS [2] [3] [4] [5] [6] [17] plus attention to bleeding that might occur with von Willebrand disease. The presence of von Willebrand domains in certain collagens [42] may account for the ability of some VWD gene mutations to cause an EDS picture.

Variants with high prevalence in normal databases [37] (barring under-diagnosis as is frequent with EDS) are assigned low relevance (VOR) and with low diagnostic utility scores are qualified as *V*ariants of *N*o *D*iagnostic *U*tility (VNODU—**Figure 2**). They will likely be registered as benign variants in the appropriate databases [37]. However, clinical judgment is required for variants like that in the profilaggrin gene (*FLG*, M135940, patient #23) that has significant prevalence (1.6%) [37], were previously associated with ichthyosis (M605803), and were judged as pathogenic for that condition by GeneDx. Their relation to AAD correlates with companion effects of *COL7A*1 gene changes on skin (patient #22), removing exoskeletal constraint and support of connective tissue. A 1% - 2% prevalence of variants disposing to AAD is compatible with the 10% - 20% of people with hypermobility [2] and dysfunction in perhaps 20% of them, recognizing that some with *FLG* gene variants will perceive only dry skin rather than arthritic or autonomic findings.

Several DNA variants among the average 30,000 in the exons of each person [43] are often highlighted as potentially significant by bioinformatic software, so variant combinations require judgment of which has primary relevance as exemplified again by patient #1 in **Table 1**—the *COL1A1* gene variant becomes primary because relevance of collagen type I genes to CTD is well-established) [41]. The accompanying sodium channel (*SCN2B* M601827) gene variant correlates with AAD-relevant variants in the same gene family (*SCN9A*-11*A* genes, patients #14 - 16), its synergistic action (VSDUS in **Figure 2**, column H) to produce AAD (as well as atrial fibrillation, M615378) upgrading the diagnostic

Table 2. Results of WES testing in patients evaluated for EDS or developmental disability.

Group	AAD	AADf	AADmm	hEDS	cEDS	BJH	DysA	NotEDS EDS	***DD***
Patients with preliminary diagnoses (% of those evaluated for EDS or DD)	1656 (100)	1337 (81)	319 (19)	1138 (69)	329 (20)	82 (5.0)	79 (4.8)	28 (1.7)	**728 (100)**
Patients with preliminary diagnoses having WES (% of those evaluated)	727 (45)	613 (47)	114 (37)#	503 (45)	160 (50)	31 (39)	29 (37)	4 (14)#	**102 (14)#**
DNA variants as reported by GeneDx[a]									
Patients having a variant (% of those having WES)	440 (61)	370 (60)	70 (61)	298 (59)	96 (60)	20 (65)	22 (76)	4 (100)#	**79 (78)#**
Total number of variants (% of total variants)	636 (100)	539 (86)	97 (16)	425 (68)	149 (24)	28 (4.5)	30 (4.8)	4 (0.64)	**131 (100)**
Patients with only a MT-DNA variant (% of those having WES)	75 (11)	63 (10)	12 (11)	46 (9.1)	18 (11)	4 (13)	6 (21)	1 (25)	**2 (2.0)#**
Number of MT-DNA variants (% of total variants)	111 (17)	94 (18)	17 (17)	71 (17)	27 (18)	5 (18)	7 (23)	1 (25)	**2 (1.5)**
Patients with nuclear DNA ± MT-DNA variant (% of those having WES)	365 (50)	307 (50)	58 (51)	252 (50)	78 (49)	16 (52)	16 (55)	3 (75)	**77 (75)#**
Number of nuclear DNA variants (% of total variants)	525 (83)	445 (82)	80 (83)	354 (83)	122 (82)	23 (82)	23 (77)	3 (75)	**129 (100)**
Patients with CTD-related likely pathogenic/pathogenic variant (“)	16 (4.4)	12 (2.0)#	4 (3.5)	9 (1.8)#	1 (0.63)#	2 (6.4)	3 (10)	1 (25)#	**1 (0.98)**
DNA variants as qualified by the author[b] in Figure 2									
Patients with variant of likely relevance to AAD[b] (% if those having WES)	148 (20)	123 (20)	25 (22)	100 (20)	33 (21)	8 (26)	6 (21)	1 (25)	**5 (4.9)#**
Number of variants likely relevant to AAD[b] (% of total variants)	169 (27)	138 (26)	31 (32)	116 (27)	36 (24)	9 (32)	7 (23)	1 (25)	**5 (3.8)**
Patients with variants previously related to CTD[c] (% if those having WES)	75 (10)	60 (9.8)	15 (13)	50 (9.9)	17 (11)	5 (16)	2 (6.9)	1 (25)	**1 (0.98)#**
Patients with variants newly related to AAD[d] (% if those having WES)	73 (10)	63 (10)	10 (8.7)	50 (9.9)	16 (10)	3 (10)	4 (14)	0	**4 (3.9)#**
Patients with variants possibly relevant to AAD[b] (% if those having WES)	227 (32)	194 (32)	33 (29)	154 (31)	49 (31)	8 (26)	13 (45)	3 (75)	**1 (0.98)#**
Number of variants possibly relevant to AAD[b] (% of total variants)	390 (61)	344 (64)	46 (47)	262 (62)	95 (64)	11 (39)	19 (66)	3 (75)	**1 (0.77)**
Patients with only VNODU or V*DUO variants[e] (% of those having WES)	65 (8.9)	53 (8.6)	12 (11)	44 (8.7)	14 (8.8)	4 (13)	3 (10)	0	**73 (72)#**
Number of VNODU or V*DUO variants[e] (% of total variants)	77 (12)	57 (11)	20 (21)	47 (7.4)	18 (12)	8 (29)	4 (1.3)	0	**123 (95)**
Maternally inherited variants of likely or possible relevance to AAD (“)	167 (32)@	147 (34)	29 (36)	110 (32)	47 (41)#	10 (43)	8 (31)	0	**25 (19)#**
Paternally inherited variants of likely or possible relevance to AAD (“)	111 (21)@	94 (21)	17 (21)	82 (23)	18 (16)	7 (30)	4 (15)	0	**28 (22)**

[a]GeneDx© qualified variants as of uncertain significance, likely pathogenic, or pathogenic; [b]variants qualified as likely—strong (VSR) or established (VER) relevance to the articulo-autonomic dysplasia (AAD) disease process, as possibly--conditional (VCR) or uncertain (VUR) relevance, or as no—(VOR) relevance; [c]DNA variants previously related to connective tissue dysplasia (CTD) as reported in the literature; [d]newly related by this study; [e]variants qualified as having no (VNODU) diagnostic utility (DU) or utility for other conditions (V*DUO, *except A for actionable rather than E for established; #percentage significantly different ($p < 0.05$) from percentage in all patients with AAD (left column) by modified chi square analysis [36] through MedCalc© (medcalc.org); @significantly different [36] at the $p < 0.001$ level; BJH, benign joint hypermobility; c, classical; DD, developmental disability; DysA, dysautonomia; f, female; h, hypermobile; m, male, MT mitochondrial, NotEDS, not diagnosed with Ehlers-Danlos syndrome.

Table 3. Numbers of genes and DNA variants found by WES in patients evaluated for EDS and developmental disability.

Group	AAD	AADf	AADm	hEDS	cEDS	BJH	DysA	NotEDS	DD
Patients with preliminary diagnoses having WES	727	613	114	503	160	31	29	4	**102**
Patients with likely relevant DNA variants in genes previously associated with CTD[a] (% of patients having WES)									
Patients[b] with *COL1A1* (6 pts)/*COL1A2* (5 pts) *COL11A1* (2 *pts*)/*COL11A2* (2 *pts*) DNA variants (4 genes)	15 (2.1)	8 (1.3)	7 (6.1)#	13 (2.6)	1 (0.63)	1 (3.2)	0	0	**0**
"with *COL3A1* (9 pts)/*VWF* (9 pts) DNA variant (2 genes)	18 (2.5)	15 (2.3)	3 (12.7)	13 (2.6)	4 (2.5)	1 (3.2)	0	0	**0**
"with *COL5A1* (20 pts) /*COL5A2* (8 pts) DNA variant (2 genes)	28 (3.9)	23 (3.8)	5 (4.4)	14 (2.8)	11 (6.9)	2 (6.5)	0	1 (25)	**1 (0.98)**
"with *FBN1* (14 pts)/*TGFB2* (3 pts)/*TGFB3* (1 pt)/ *TGFBR1* (2 pts) *TGFBR2* (3 pts) DNA variant (5 genes)	23 (3.2)	21 (3.4)	2 (1.8)	16 (3.2)	3 (1.9)	2 (6.5)	2 (6.9)	0	**0**
Patients with likely relevant DNA variants in genes associated with AAD by this study[c] (% of patients having WES)									
"with *SCN9A* (6 pts)/*SCN10A* (5 pts)/*SCN11A* (3 pts) *POLG* (10 pts) DNA variant (4 genes)	24 (3.3)	22 (3.6)	2 (1.8)	18 (3.6)	6 (3.8)	0	0	0	**2 (2.0)**
"with *COL6A1* (4 pts)/*COL5A2* (1 pt)/*COL6A3* (3 pts)/*COL12A1* (11 pts) DNA variant (4 genes)	19 (2.6)	15 (2.4)	4 (3.5)	12 (2.4)	4 (2.5)	2 (6.5)	1 (3.4)	0	**0**
"with *COL7A1* (4 pts)/*FLG* (17 pts) DNA variant (2 genes)	21 (2.9)	19 (3.1)	2 (1.8)	14 (2.8)	4 (2.5)	0	3 (10)#	0	**2 (2.0)**

[a]DNA variants in genes judged by the author as having strong or established relevance to the articulo-autonomic dysplasias (AAD) disease process (**Figure 2**), having a previously reported association with connective tissue dysplasia (CTD; [b]numbers of patients with variants in each gene shown in parentheses; [c]variants of likely relevance, newly related to the AAD process by this study—the von Willebrand factor *VWF* gene variants are newly related but are grouped with *COL3* gene variants because of their vascular impact; #percentage significantly different ($p < 0.05$) from percentage in all patients with AAD (left column) by modified chi square analysis [36] through MedCalc© (medcalc.org); BJH, benign joint hypermobility; c, classical; *COL*, collagen; DD, developmental disability; DysA, dysautonomia; f, female; *FBN1*, fibrillin-1; *FLG*, profilaggrin; h, hypermobile; m, male; NotEDS, not diagnosed with Ehlers-Danlos syndrome; *SCN/POLG*, sodium channel/polymerase gamma; *TGFB*, transforming growth factor beta; *VWF*, von Willebrand factor.

utility for the variant combination to strong (3+ or VSDU) in **Table 1**. The additional potassium channel *KCNH2* (M152427) gene variant in patient #1 has been observed previously in a patient with a different arrhythmia (long-QT syndrome, M613688) and must be qualified as having conditional diagnostic utility for another diagnosis (VCDUO, **Figure 2**). The resulting clinical diagnosis for patient #1 takes into account the many typical historical (48 of 80) and physical (20 of 40) findings of AAD [17] and classical EDS (more skin findings—4 of 5 including scarring but less hypermobility—7 of 12, left columns, **Table 1**) rather than osteogenesis imperfecta [41], the additional variants mandating monitoring for cardiac and neuromuscular changes from sodium channel alterations [44] in addition those of AAD (right column, **Table 1**).

Even when qualified as having actionable (VADU-4+) *diagnostic utility*, a DNA variant or variant combination should contribute to a *clinical* diagnosis, not the *molecular* one promoted by some [38], *if* medical knowledge ensures compatibility with usual inheritance and patient findings (IFClin columns in **Figure 2**). The complex route from variant diagnostic utility to clinical diagnosis is first illustrated by the 287 (40%) of 727 patients with typical AAD findings who were found to have no variants of possible pathogenic significance and the 65 (8.9% of 727) with variants of no diagnostic utility (VNODU) or relevance to

other diseases (V*DUO—Table 2). The negative genomic analysis neither contributes to nor excludes the clinical diagnosis in this period of gene discovery (upper panel, ClinDx column, Figure 2), especially for a multifactorial disorder like AAD but also for most Mendelian diseases as well since deep sequencing [45] of intergenic regions, particularly those encoding microRNAs or the 6% consisting of conserved non-coding elements (CNEs, conserved from humans to fish) [24] [46] remains embryonic.

DNA testing. Table 2 summarizes the results of whole exome sequencing with mitochondrial DNA analysis as performed through GeneDx (see Methods) on 727 (45%) of the 1656 patients referred for evaluation of EDS from 2011-18. Significantly [36] fewer males with their milder disease had WES testing along with those not diagnosed with EDS or the comparison developmental disability group with its preliminary chromosome studies (Table 2, upper rows). At least one DNA variant was reported in the 440 patients referred for evaluation of EDS and subsumed as AAD (61% of the 727 tested, left column), those preliminarily diagnosed as EDS types (hypermobile, classical), benign joint hypermobility, or with more dysautonomia having similar proportions (subsequent columns, Table 2). Higher percentages of patients not thought to have EDS (100% of the four having WES) or those with disability (78%) had at least one DNA variant.

Because 120 of the 440 EDS patients with a potentially significant DNA change reported by GeneDx had multiple variants, numbers of patients and DNA variants must be tallied separately in Table 2. There were 636 DNA variants in all, 365 patients (50% of 727) having 525 variants (83% of 636) in nuclear genes (21 with additional mitochondrial DNA variants), 75 patients (11% of 727) having only mitochondrial variants, yielding 111 variants (17% of 636) in mitochondrial DNA (Table 2; some of the 96 patients had more than 1 mitochondrial DNA variant). Not included were 14 patients who had prior testing using panels of genes related to CTD, 2 of them (14%) yielding variants reported pathogenic for CTD by GeneDx, a proportion not significantly different from WES results as qualified by GeneDx or the approach in Figure 2 (see Discussion).

Similar proportions of patients with different preliminary diagnoses (female, male, EDS hypermobile, classical, or benign hypermobility) had nuclear (49% - 52%) or single mitochondrial DNA (9.1% - 13%) variants, the higher proportions of 55% nuclear and 21% mitochondrial for dysautonomia patients not statistically significant (Table 2). Only 16 patients (4.4%) had variants qualified as likely pathogenic/ pathogenic and related to CTD by GeneDx (Table 2, middle rows), proportionally less in females (2.0%) or major EDS types (1.8% to 0.63%) but higher in males (3.5%) and those with benign joint hypermobility (6.4%) or dysautonomia (10%, some numbers statistically significant, Table 2). Most variants qualified as likely/pathogenic by GeneDx had diagnostic utility for other diagnoses (V*DUO) using the qualifications in Figure 2, conferring carrier status as with a variant in one hemochromatosis-1 (*HFE*, M613609) gene copy in patient #9 of Table 1 or having diagnostic utility for diseases screened as sec-

ondary findings [34]—one patient with a *BRCA*1 (M113705) variant, two with *BRCA*2 (M600185), three with two-copy variants in the *HFE* gene, and one with a variant in the *PMS*2 gene associated with colon cancer (M614337).

Table 2 (middle rows) shows that 148 patients (20% of 727) had variants of likely (VSR, VER) relevance to AAD, 227 (32% of 727) variants of possible (VUR, VCR) relevance to AAD including those with mitochondrial DNA variants, and 65 patients (8.9% of 727) variants of no diagnostic utility (VNODU) or utility for diagnosis of other diseases (V*DUO). The proportions of patients having variants with likely relevance to AAD were very similar (20% - 22%) among males, females, or patients with preliminary diagnoses of hypermobile or classical EDS. Only benign joint hypermobility patients (8 of 31, 26%) showed statistically insignificant differences.

Numbers of variants and genes paralleled patient numbers with 169 variants (27%) likely, 390 (61%) possibly, and 77 (12%) not relevant to AAD out of 636 total (Table 2), altering respectively 23 (9.2%), 160 (64%), and 67 (27%) of 250 total genes (data not shown). Proportions of likely relevant variants are again similar between groups (23% - 32%) but proportions of genes varied significantly (11% - 42%, $p < 0.05$ among groups, data not shown) because of group size and the fact that the same gene can be altered in different groups and counted multiple times. Of importance here are the variants linking 23 genes to the AAD process as shown in Table 2 and Table 3.

Among the patients with variants of likely relevance to AAD were 75 patients (10% of 727 in Table 2) with changes in 12 genes traditionally associated with connective tissue dysplasia (e. g., in the *COL*1*A*1 gene-M120150—upper group in Table 2) and another 73 patients (also 10% of 727 in Table 2) with DNA variants in 11 genes newly associated with AAD (e.g., in the *POLG* gene-M174763, lower group of Table 3). The 148 patients with likely relevant variants are grouped by gene action inferred by prior disease associations—*COL*1, 5, 11, *FBN*1, and *TGFB* genes on generalized connective tissue matrix, *COL*3 *and VWF* genes on vessel, *SCN/POLG* on nerve, *COL*6, 12 on muscle, and *COL7A*1, *FLG* variants on skin as mentioned previously. Information on each gene and their associated diseases are in the right column of Table 1; history and physical findings on these example patients as detailed previously [17] are listed in the left columns.

Table 3 shows numbers of gene variants considered of primary import to diagnostic utility and omits 21 additional variants in these 23 genes that occurred in combination and were qualified as V*DUS. Significant numbers of classical EDS patients (11% or 6.9%) had *COL*5 gene variants as expected [8], significantly [36] more than hypermobility EDS [3] patients (14% or 2.8%, $p < 0.05$), although few subgroup proportions differed significantly from those of AAD patients as a whole (Table 3). These relatively equal distributions of individual gene changes among patient groups emphasize that changes in particular genes do not correspond to EDS types at least as defined by preliminary diagnosis here (see Methods for criteria); they also refute prevalent assumptions that patients

with hypermobile EDS do not have gene changes. Note that the one patient (patient #7 in Table 1) with a *COL5A*1 gene variant who did not meet criteria for diagnosis of AAD or EDS as listed in Table 3 had conditional diagnostic utility (VCDU), consistent with the non-diagnosis pending the occurrence and qualification of that variant in future patients.

Giving separate validation to the qualification of 375 EDS patients (52% of 727) as having DNA variants of likely or possibly relevance are the significantly ($p < 0.05$) lower numbers (4.9% or 0.98%) of 102 developmental disability patients so qualified in Table 2. There were 4 genes altered in both groups that share potential relevance to CTD: 2 disability patients had variants in the polymerase gamma (*POLG* M174763) gene, 2 in the profilaggrin (*FLG*, M135940) gene, 1 in the collagen type XI alpha-1 chain (*COL*11*A*1, M120050) gene, and 1 in the procollagen lysyl oxidase (*PLOD*1, M153454) gene. Shared genes with relevance to other disease included the L1 cell adhesion molecule *L*1*CAM* gene (M308840) on the X chromosome, one conferring carrier status in a female with EDS, the other explaining symptoms in a boy evaluated for significant intellectual disability. Only 1 patient with disability (0.98%) had a mitochondrial DNA variant compared to 13% of AAD patients (Table 2), reflecting referral of patients with disability and likely mitochondrial disease [47] to other subspecialists.

Roughly equal numbers of maternally (25, 19%) versus paternally (28, 22%) inherited variants among 129 in disability patients contrasted with the respective 167 (32%) from mothers and 111 (21%) from fathers among 525 in AAD patients (lower rows, Table 2—differences highly significant) [36]. Many more *de novo* variants (41% or 32%) occurred in disability than AAD patients (7% or 1.3%, data not shown), correlating with their developmental impact. This significant excess of maternally inherited variants correlates with the excess maternal transmission of AAD documented in Figure 1 and the many mitochondrial DNA variants shown in Table 2.

4. Discussion

Fundamental to clinical appreciation of WES results is recognizing the progression from disposition [31] to distress [13] [14] [15] to disease [2] [3] [4] [5], a progression arising from myriad connections of genes [48] and tissues during development that ensure individual flavors of pathogenesis. Inevitably then is a discovery phase of WES testing that filters candidate gene variants from the 30,000 that alter protein among the 2 - 3 million in each person [43] and elects relevance based on prevalence in ostensible health versus disease [37]. Because both categories can include the disposed or distressed, clinical perspective must determine whether a DNA variant travels with [31], predicts [28], or has relevance [33] to disease diagnosis through an approach like that in Figure 2. Novel DNA changes should not be excluded from consideration by lack of functional analysis (column Fa, Figure 2) because the latter 1) is available only in research laboratories, 2) rarely uses patient tissue with its unique, polygenic background,

and 3) cannot replicate *in vivo* pathophysiologic mechanisms like articulo-autonomic dysplasia.

Advantages of the clinical approach outlined in **Figure 2** include the connotations of diagnostic utility grades like *VNODU*, *VUDU* and *VADU* (Veda) that emphatically convey clinical irrelevance or validation; the right columns emphasize that a DNA change never makes a specific medical diagnosis until the doctor code is applied. Medical judgments of pleiotropy (one gene, several diseases), penetrance (expression of symptoms), and specificity (which disease) must be imposed before a DNA variant becomes clinically diagnostic as connoted by the *IFClin* columns of **Figure 2**. DNA variation conveys *utility* rather than *certainty of* diagnosis as some have claimed [38], shown by patients with homozygous glutamic acid to valine in β-globin mutations who do not develop sickle-cell anemia [49]. Relevance to disease *process* should then be decided, following consensus guidelines [33] by promoting triply recurring variants for established use in DNA testing and recycling those that are not (VOR) to determine diagnostic utility for other pathology (VNODU, V*DUO in **Figure 2**).

Diagnostic utility of the most relevant (primary) DNA variant is increased by patient findings expected for the process in question and synergistic action of additional (secondary) variants (**Figure 2**, column H), utility of the variant or variant combination and its correlation with family (column I) and specific disease findings (column Fd) supporting a clinical diagnosis (ClinDx). Similar proportions of patients with hypermobile or classical EDS, benign joint hypermobility, or dysautonomia (20% - 26%, **Table 2**) had variants in genes of likely relevance to AAD, supporting membership of these sub-types in an AAD category and suggesting common action of the implicated collagen, fibrillin, polymerase gamma, sodium channel, transforming growth-factor, and von Willebrand genes to produce an AAD profile [17] that includes common EDS types.

Autosomal dominance expected for CTD is suggested by the 279 3-generation families with findings of AAD/EDS described in Results, but the 96 patients with mitochondrial DNA variants and excess of female transmission in **Table 2** suggests maternal influence. The idea of mitochondrial dysfunction contributing to AAD is intriguing for further investigation, supported by the 10 patients with mitochondrial DNA polymerase gamma (*POLG*) variants (grouped with 14 having *SCN* gene variants in **Table 3**) but opposed by the absence of optic disc/retinal findings, hearing deficits, ataxia, or biochemical findings (elevated plasma lactate, Krebs cycle intermediates) [47] in the 3 *POLG* and 2 mitochondrial DNA variant patients who had such studies (data not shown).

The cycle of articular and autonomic dysplasia proposed previously [17] can now be elaborated to show how changes in relevant genes act to enhance tissue laxity (upper right side of **Figure 3**) and/or autonomic imbalance (lower left side of **Figure 3**). Genes with impact on cartilaginous/osseous, cutaneous, or general connective tissue elements enhance articular dysplasia, its vessel laxity and lower body pooling eliciting sympathetic stimulation (upper right, **Figure 3**). Because

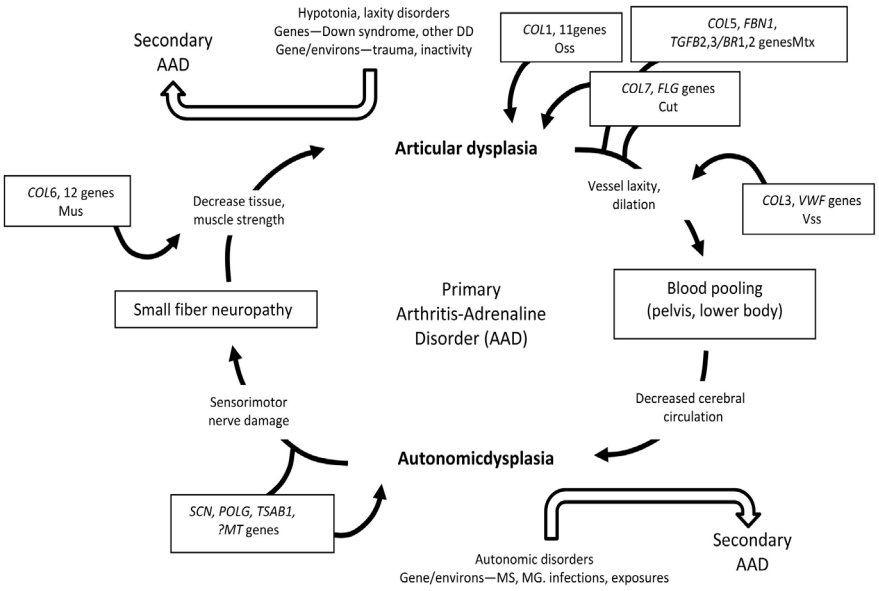

Figure 3. Influence of various gene alterations on the articulo-autonomic dysplasia cycle. AAD, for both the process of articulo-autonomic dysplasia and its clinical result, arthritis-adrenaline disorder; COL, collagen; Mtx, joint-tissue matrix impact; Cut, cutaneous impact; DD, developmental disabilities; FBN1, fibrillin 1; FLG, profilaggrin; HSAN, hereditary sensory autonomic neuropathies; MG, myasthenia gravis; MS, multiple sclerosis; MT, mitochondrial genes; Mus, muscular impact; Nrv, nerve impact; Oss, osseous impact; POLG, polymerase gamma; SCN, sodium channel, voltage-gated, type IX to XI, alpha-subunit; TPSAB1, tryptase alpha-beta 1; TGFB2, 3/TGFBR1, 2, transforming growth factor, beta-2 and 3/transforming growth factor beta-receptor type 1 and 2; Vss, vascular impact; VWF, von Willebrand factor.

many collagens are distributed throughout connective tissue, interlinked and dependent on one another for assembly [48], disproportionate impact on bone or skin is not meant to exclude action on other tissue elements.

Alterations of collagen type III that impact cardiovascular development [48] can be placed nearer the vessel laxity portion of the cycle (**Figure 3**, right), grouped with interacting von Willebrand factor [42] as having vessel impact. Extremely important are findings in patients with *COL3* gene variants that are typical of common EDS types [2]-[8] rather than the chiseled face, bulging eyes, multiple aneurysms, bowel ruptures, and pregnancy complications of vascular EDS [9]. The only aneurysm documented in thorough vascular surveys performed on 6 of the 9 patients (example patient #6 in **Table 1**) and on 3 of their relatives with *COL3* gene variants was one affecting the iliac artery in the father of one patient.

Entering on the autonomic side of the AAD cycle are alterations of *SCN/POLG* genes with impact on small nerve fibers [44], acting along with *COL6/*12 variants to decrease intrinsic and surrounding muscle strength (left side, **Figure 3**) [50]. These genes with impact on muscle highlight a myopathic category [51] [52] of EDS that needs further study but will likely encompass many patients now classified as hypermobile EDS [4]. A predominant myopath-

ic category would correlate with AAD symptoms in many non-CTD conditions that exhibit hypotonia/poor muscle development [53] and explain why 6 patients with developmental disabilities had DNA variants of relevance to AAD in Table 2. Less muscle support and protection leading to joint-tissue laxity would explain the established benefits of exercise [54] and physical therapy [55] for EDS. Impact on the various tissue elements shown in Figure 3 adds focus on patient-derived stem cells that could repair multiple mesodermal tissues [56] [57], their gene defects characterized by WES and perhaps corrected by emerging technology [58].

Future research on AAD and its component or ancillary disorders can be envisioned by the diagram in Figure 4. Patients having the reciprocal hypermobility and autonomic findings suggestive of EDS are preliminarily diagnosed as arthritis-adrenaline disorder (center), *then* assigned specific diagnoses based on correlation of genomic and other laboratory/imaging results (example patients in Table 1, right column). The 52% of patients with AAD-relevant variants reported here (Table 2) suggest that WES could reach a 70% or higher yield of diagnostically useful variants for EDS patients, limited by poor detection of aggregate effects from minor DNA variations [59] and unexplored intergenic variation that requires deep sequencing [45]. Familiar gene changes will have diagnostic utility for known EDS types (Figure 4, left lower square) while new ones like many reported here will have utility for the emerging myopathic EDS (lower center square) and other new types [44] associated with genes causing dysautonomia (lower right square).

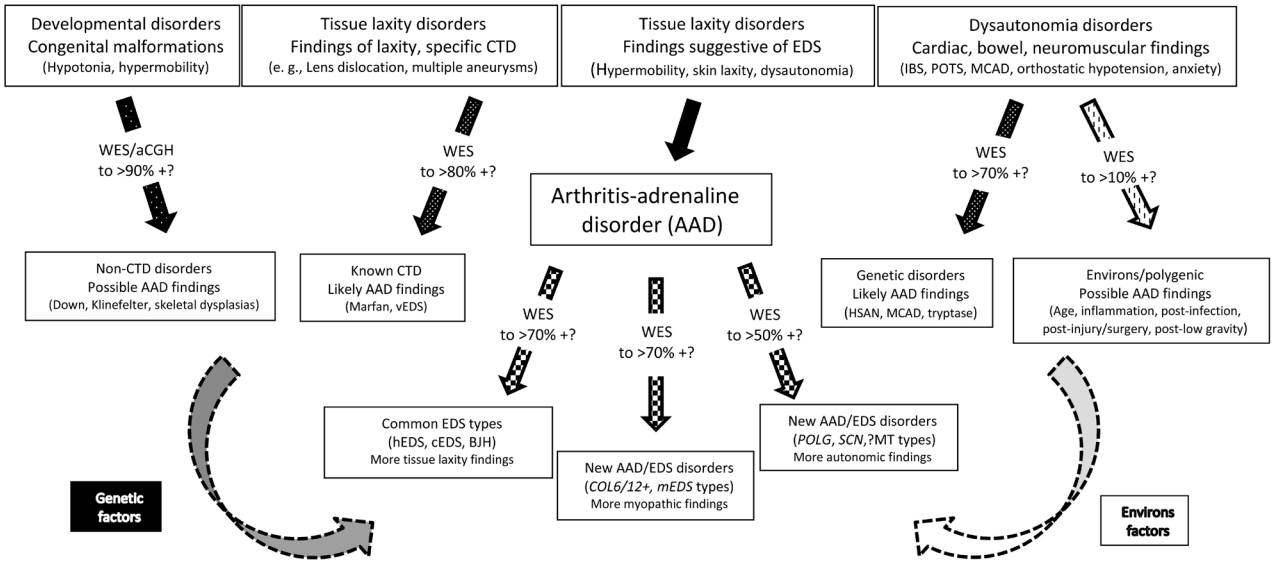

Figure 4. Diagnostic approach to diseases with articulo-autonomic findings. Disease categories are shown above and below the arrows with their predicted yields from whole exome sequencing (WES); aCGH, array-comparative genomic hybridization; BJH, benign joint hypermobility; COL, collagen gene, CTD, connective tissue dysplasia; c, h, m, vEDS—classical, hypermobile, myopathic, vascular EDS (Ehlers-Danlos syndrome); FLG, profillagrin gene, HSAN, hereditary sensory autonomic neuropathy; IBS, irritable bowel syndrome; MCAD, mast cell activation disorder; POLG, polymerase gamma gene; POTS, postural orthostatic tachycardia syndrome; SCN, sodium channel gene.

Preliminary diagnosis of patients with AAD and differentiating findings like lens dislocation will focus on specific CTD disorders (**Figure 4**, left center) like Marfan syndrome (M150700), their strong genetic predisposition predicting higher genomic testing yields. The AAD process as a secondary complication can be anticipated in a wide range of disorders, often unrecognized in congenital disorders with malformations, hypotonia, and joint laxity (far left) [53] that should have high yields from genomic testing (as shown by the 78% of tested developmental disability patients having variants in **Table 2**). On the near right are genetic causes [12] of dysautonomia and far right are diverse conditions with autonomic imbalance, most multifactorial like aging, post-infection, re-adjustment to gravity [60] (right square), and other acquired disorders [61] where protein-coding changes will imply disposition rather than disease.

A primary goal of future research is to couple molecular (left panels, **Figure 2**) and Mendelian insights (middle panels) to medical knowledge (right panels) if genomic analysis is to reach its potential for prevention and tailored therapy [62]. Given these present and future possibilities, knowing the name and genomic nature [1] of AAD can keep its many predispositions from blighting the cradle [28] and at least some of its victims from an untimely grave.

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Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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